Production of cytidine 5′-monophospho-*N*-acetyl-β-D-neuraminic acid (CMP-sialic acid) using enzymes or whole cells entrapped in calcium pectate–silica-gel beads

Jozef Nahálka*[1], Bingyuan Wu†, Jun Shao‡, Peter Gemeiner* and Peng George Wang§

Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, SK-84538 Bratislava, Slovak Republic, †Neose Technologies Inc., 102 Witmer Road, Horsham, PA 19044, U.S.A., ‡Symyx Technologies Inc., 3100 Central Expressway, Santa Clara, CA 95051, U.S.A., and §Department of Biochemistry, Ohio State University, 484 W 12th Ave, Columbus, OH 43210, U.S.A.

The present study focuses on the application of immobilization technology to enzymic sugar syntheses. The paper describes an improved silica–alginate matrix established for entrapment and encapsulation. The replacement of alginate with pectate provided enhanced chemical resistance of the matrix, which allows the use of 1% (w/v) polyphosphate in reaction mixtures. Polylysine, a reagent for silica condensation, was replaced by a much cheaper alternative, namely polyethyleneimine. The proposed design was applied in the production of cytidine 5′-monophospho-*N*-acetyl-β-D-neuraminic acid (CMP-sialic acid) by immobilized recombinant enzymes or *Escherichia coli* cells containing overexpressed enzymes. A comparison between these two strategies was made. On the basis of the results we conceptualized a system to synthesize sialyloligosaccharides by using a biocatalyst entrapped in calcium pectate–silica gel beads.

Introduction

Immobilization of enzymes or whole cells in a suitable matrix is an important practice in the economical utilization of biocatalysts. The composition and configuration of the matrix material, whether of natural or synthetic origin, contribute to the efficiency of the biocatalyst in a given application. Calcium alginate gel (CAG) has been widely used in cell entrapment and encapsulation during the last three decades. Alginate defines an extracellular environment that is beneficial to cell viability and function, and it even serves as a protection that provides for prolonged enzymic activity of permeabilized cells. However, CAG beads cannot provide satisfactory results in many biotechnological applications because of their sensitivity to calcium-chelating compounds, especially phosphates and citrates. Calcium pectate gel (CPG) resembles alginate in sol → gel transition behaviour, but it is much less sensitive to the ions and chemical agents that break down CAG. It has been shown that the replacement of alginate with pectate maintains diffusion properties of the gel matrix while improving its mechanical and chemical properties [1,2]. The stability of CAG can also be improved by additional coating with polycation and/or cross-linking [3,4]. Moreover, the coating step improves the efficiency of enzyme immobilization by decreasing the pore size of the beads to prevent enzyme leakage. Increasing the size of catalytic particles is another choice for enzyme entrapment when the gel lattice is not dense enough [5,6]. In the present study we combined all these strategies and designed a calcium pectate–silica gel for whole cell and enzyme immobilization. The immobilized preparations were then applied in multi-step synthesis of cytidine 5′-monophospho-*N*-acetyl-β-D-neuraminic acid (CMP-sialic acid).

Sialic acid is a terminal constituent of glycoconjugates that plays crucial roles in cell–cell recognition. Sialyl epitopes are thought to be target carbohydrate antigens in various immunotherapy applications (e.g. cancer immunotherapy) [7]. CMP-sialic acid, as activated sialic acid, is the substrate of sialyltransferases, key enzymes for the enzymic preparation of sialylated biomolecules [8]. So far two methods have been employed in biocatalytic synthesis of CMP-sialic acid. Enzymic synthesis is accomplished in relatively 'clean' reactions without the degradation of product and the generation of by-products. Whole-cell reactions, on the other hand, eliminate the tedious multiple

Key words: cytidine 5′-monophospho-*N*-acetyl-β-D-neuraminic acid (CMP-sialic acid), enzymic synthesis, hydrogel, immobilization.

Abbreviations used: CAG, calcium alginate gel; CMP-sialic acid, cytidine 5′-monophospho-*N*-acetyl-β-D-neuraminic acid; CMP, cytidine 5′-monophosphate kinase (EC 2.7.4.14); CPG, calcium pectate gel; P_n, polyphosphate; LB medium, Luria–Bertani medium; PEI, polyethyleneimine; APS, alginate–polylysine–silicate microcapsules; AS, alginate–silica mixed gel; PSPS, pectate–silicate–PEI–silicate particles; ManNAc, *N*-acetyl-D-mannosamine; NanA, *nanA* gene product (sialic acid aldolase, EC 4.1.3.3); NeuA, *neuA* gene product (CMP-sialic acid synthetase, EC 2.7.7.43); EP, enzyme preparation; CP, whole-cell preparation; NTP, nucleoside 5′-triphosphate.

* To whom correspondence should be addressed (email nahalka@savba.sk).

enzyme-purification steps. Therefore it is timesaving and economically favourable. In the present study we tried to compare the performance of these two different approaches.

Materials and methods

Materials

Potassium pectate (from the Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic) was prepared from commercial apple pectin by de-esterification in heterogeneous phase under strictly controlled conditions to minimize pectin backbone degradation (M_r 165 000, 81 % D-galacturonate, degree of esterification = 0). PEI (polyethyleneimine, $\overline{M}_w \approx 2000$), sodium alginate and sodium silicate solution ('water glass') were from Aldrich (Milwaukee, WI, U.S.A.). *Escherichia coli* K-12 (A.T.C.C. 47076) and *E. coli* K1 (A.T.C.C. 13027) were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). *E. coli* DH5α (lacZΔM15 hsdR recA) was from Gibco–BRL Life Technology. *E. coli* BL21(DE3) [*F⁻ ompT hsdS$_B$*($r_B^- m_B^-$) *gal dcm* (*DE3*)] and plasmid DNA pET15b were bought from Novagen (Madison, WI, U.S.A.). All kits for DNA isolation and purification were from Qiagen (Valencia, CA, U.S.A.). All restriction enzymes, 1 kb DNA ladder, and T_4 DNA ligase were obtained from Promega (Madison, WI, U.S.A.). Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA, U.S.A.). Polyphosphate (polyphosphoric acid sodium salt, P_n) and other chemical reagents were from Sigma.

Recombinant enzymes and cells

nanA and *neuA* genes [producing the products NanA (sialic acid aldolase) and NeuA (CMP-sialic acid synthetase)], amplified by PCR, were digested with restriction endonucleases *Xba*I/*Sal*I and *Sal*I/*Bam*HI and ligated tandemly into pET15b linearized with *Xba*I/*Bam*HI.

E. coli was cultivated in LB (Luria–Bertani) medium (10 g/litre tryptone, 5 g/litre yeast extract and 10 g/litre NaCl). Freshly transformed *E. coli* BL21(DE3) harbouring the recombinant plasmid was grown in LB medium (50 ml) with ampicillin (150 μg/ml) overnight (30 °C, 225 rev./min), then transferred into fresh LB medium (1 litre) with ampicillin for another 3 h at 37 °C. When the attenuance (D_{600}) reached 0.8–1.0, the culture was induced with 400 μM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 4 h at 37 °C. The cells were harvested by centrifugation (4500 **g**, 10 min, 4 °C), disrupted by sonication on ice in 25 ml of binding buffer [20 mM Tris/HCl (pH 7.9)/0.5 M NaCl/5 mM imidazole], clarified by centrifugation and loaded on to an Ni^{2+}-nitrilotriacetate column (Qiagen). The column was first washed with binding buffer [20 mM Tris/HCl (pH 7.9)/0.5 M NaCl/5 mM imidazole], and then with washing buffer [20 mM Tris/HCl (pH 7.9)/0.5 M NaCl/20 mM imidazole], followed by elution with elution buffer [20 mM Tris/HCl (pH 7.9)/0.5 M NaCl/200 mM imidazole]. The eluates were concentrated by ultrafiltration, dialysed against 20 % (v/v) glycerol and stored at 4 °C. The whole-cell paste was stored at − 20 °C.

Immobilization

The enzyme solution or suspension of *E. coli* BL21(DE3) cells containing overexpressed enzymes, potassium pectate or sodium alginate, and sodium silicate, were mixed at pH 7.5–8.5 to the following final concentrations: enzymes, 0.5–1.0 mg/ml, or cells, 5–10 mg/ml; alginate, 2 % (w/v), or pectate, 5 % (w/v); silicate, 0.4 %. Spherical-particle formation was achieved by dripping the suspension through a capillary (300 μm internal diameter) into stirred precipitation solution (0.1 M $CaCl_2$, pH 7.5–8.5) using coaxial nitrogen flow. The particles were incubated in a precipitation bath for 40 min at 4 °C. Afterwards, the beads (diameter 750–1050 μm; mean 900 μm, S.D. 97.4 μm) were stabilized by treatment with 0.25 % (w/v) PEI (40 min) and subsequently with 30 mM silicate (60 min, 4 °C, pH 7.5–8.5) or 1 % (v/v) glutaraldehyde (1 min). The stability of the beads was tested in a solution mimicking nucleotide-sugar synthesis [1 % (w/v) P_n/50 mM $MgCl_2$, pH 7.8]. The tests were performed with 50 beads in 20 ml at 23 °C on rotary shaker (30 rev./min). The mechanical resistance was measured as a function of loading (g) and time (s) required to compress the gel beads to 50 % of their original sizes [1].

Enzymic synthesis

The reaction mixture contained immobilized biocatalysts, substrates, P_n and other required ingredients. Precise compositions are described in the legends to the Figures. The small-scale (500 μl) reactions were performed at 23 °C with shaking at 30 rev./min, and 100–200-ml-volume reactions were performed at 30 °C and 150 rev./min.

Analysis

The concentration of CTP, CDP, CMP and CMP-sialic acid were measured by capillary electrophoresis (ISCO model 3850 Capillary Electropherograph). Samples were introduced into an unmodified silica column (75 μm internal diameter; 50 cm total length; 35 cm to detector) with 2 s vacuum injection. The running buffer was 25 mM sodium tetraborate, pH 9.4. Electrophoresis was carried out at 250 V · cm^{-1}, 40 μA and at 23 °C, with the detector set at 262 nm and 0.05 AUFS (absorbance units full scale).

Results and discussion

Immobilization

Alginic acid and associated salts, the alginates, are important components of the cell walls of brown algae (Phylum Phaeophyta). It has been shown that silicon is involved in the stability and rigidity of the cell wall in some algae, especially diatoms (Phylum Bacillariphyta). The polypeptides and long-chain polyamines of diatoms were identified as mediating the formation of silica nanospheres *in vitro* without extremes of temperature, pressure or pH normally required for the geological and chemical synthesis of glass and other siloxane-based materials [9]. Coradin et al. [3] simply mimicked this natural pathway and designed alginate–polylysine microcapsules coated with silicate [APS (alginate–polylysine–silicate microcapsules)] for bioencapsulation. They utilized β-galactosidase as a model to study the encapsulation, in which the enzyme was too large to leak out during the gelation of alginate droplets in $CaCl_2$ solution. We focused on recombinant enzymes that were of molecular mass less than 70 kDa, so that an increase in enzyme size was needed. Plant physiologists and biochemists have worked on plant samples and shown that silicon effectively flows in the sap as a protein chelate [10]. The chelating and/or adsorption properties of silica were used to immobilize various proteins in a mixed gel composed of colloidal silica and alginate (AS) [6]. The AS gel held proteins independent of their molecular size.

Pectins, pectinates and pectates are important constituents of the cell wall and soft tissues of higher plants; they are, in many ways, the 'land-based' counterparts of alginate in sea plants. Pectate is not only an alternative to alginate, but also the stability constant of calcium pectate is almost one order of magnitude higher than that of calcium alginate [1]. To immobilize the recombinant enzymes, we designed a calcium pectate–silica gel coated with PEI and silica [PSPS (pectate–silicate–PEI–silicate particles)]. PEI, a weak polybasic aliphatic amine, is the substitute for polylysine used by Coradin et al. [3] for silica condensation. The resistance of the beads to P_n was tested in a solution containing 1% (w/v) P_n and 50 mM Mg^{2+} at pH 7.8 (Figure 1). This mimics the reaction conditions of glycosylation catalysed by Leloir glycosyltransferases where P_n is used as energy source. The alginate gel was very unstable, and it broke down within a few minutes. By contrast, the pectate gel was stable, although some significant changes in the mechanical resistance of the gel were observed (Figure 1b). Surprisingly, coating with PEI lowered the mechanical resistance of the calcium pectate/silica mixture at zero time (Figure 1a). However, long-term storage showed that the PEI coating was critical to the stability of the pectate/silica mixture in P_n solution, and additional silica coating improved the stability. PEI cross-linking with glutaraldehyde gave the best stability; however, it negatively influenced the activities of immobilized enzymes (results not shown). Therefore glutaraldehyde cross-linking was applicable only for whole-cell immobilization. Generally speaking, calcium pectate/silica gels showed a better strength, but also a higher brittleness, compared with gels lacking silica.

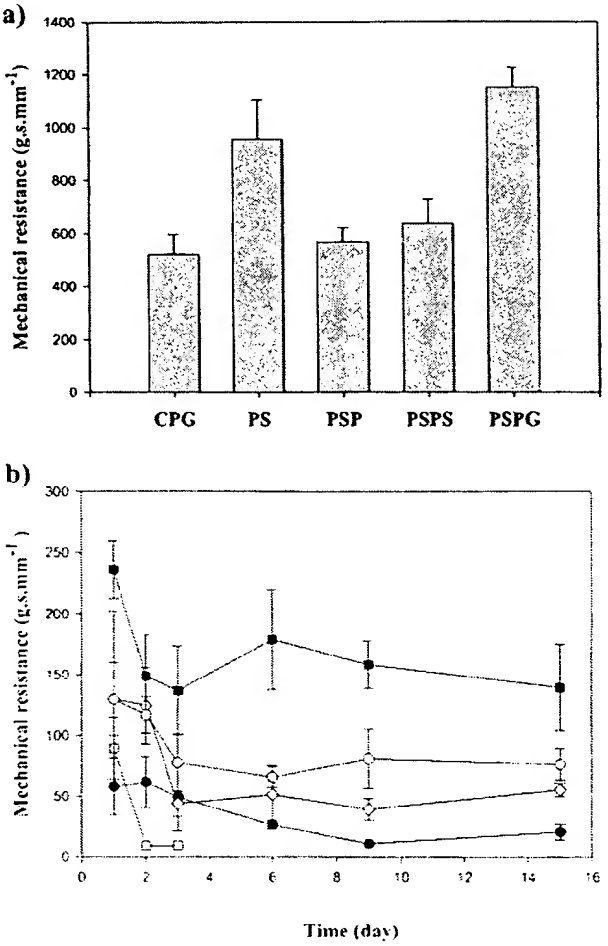


Figure 1 (a) Mechanical resistance of the pectate gels at zero time and (b) the storage stability of pectate gel beads

(a) CPG, calcium pectate/silica mixture (PS), calcium pectate/silica mixture coated with polyethylenimine (PSP), PSPS and PSPG. (b) CPG (●), PS (□), PSP (×), PSPS (○) and PSPG (■). The storage stability of gel beads was tested in 1% (w/v) P_n/50 mM $MgCl_2$, pH 7.8.

Enzymic synthesis of CMP-sialic acid

Sialic acid is synthesized from *N*-acetyl-D-mannosamine (ManNAc) and pyruvate by sialic acid aldolase (NanA; EC 4.1.3.3). The activation of sialic acid by CTP to generate the nucleotide-sugar is catalysed by CMP-sialic acid synthetase (NeuA; EC 2.7.7.43). *E. coli nanA* and *neuA* genes were amplified by PCR and cloned tandemly into pET15b vector. Both enzymes were overexpressed in

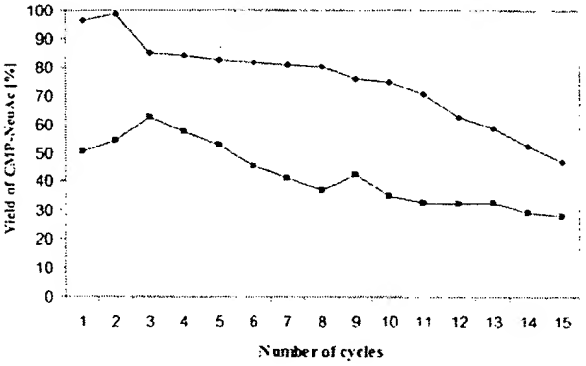


Figure 2 Recyclability of NeuA and NanA enzymes (◆) and whole cells (containing overexpressed NeuA and NanA) (■) immobilized in PSPS

The reaction was performed in a volume of 0.5 ml containing 50 mM Tris/HCl, pH 7.8, 50 mM $MgCl_2$, 0.25 % Triton X-100, 1 % (w/v) P_n, 20 mM CTP, 30 mM ManNAc, 75 mM pyruvate and 100 mg of PSPS or PSPG (pectate–silicate–PEI–glutaraldehyde particles). The enzyme activity at t_0 was 251 munits/g for the 24 h cycle and 103 munits/g for the 48 h cycle. CMP-NeuAc ≡ CMP-sialic acid

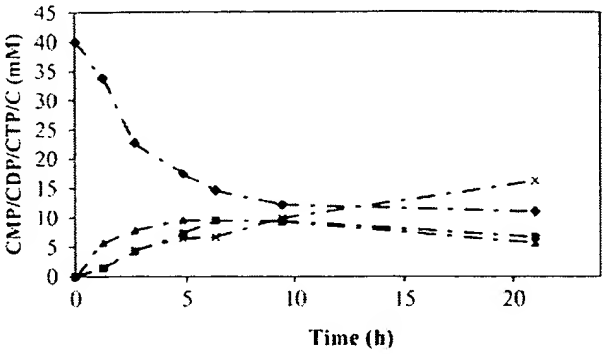
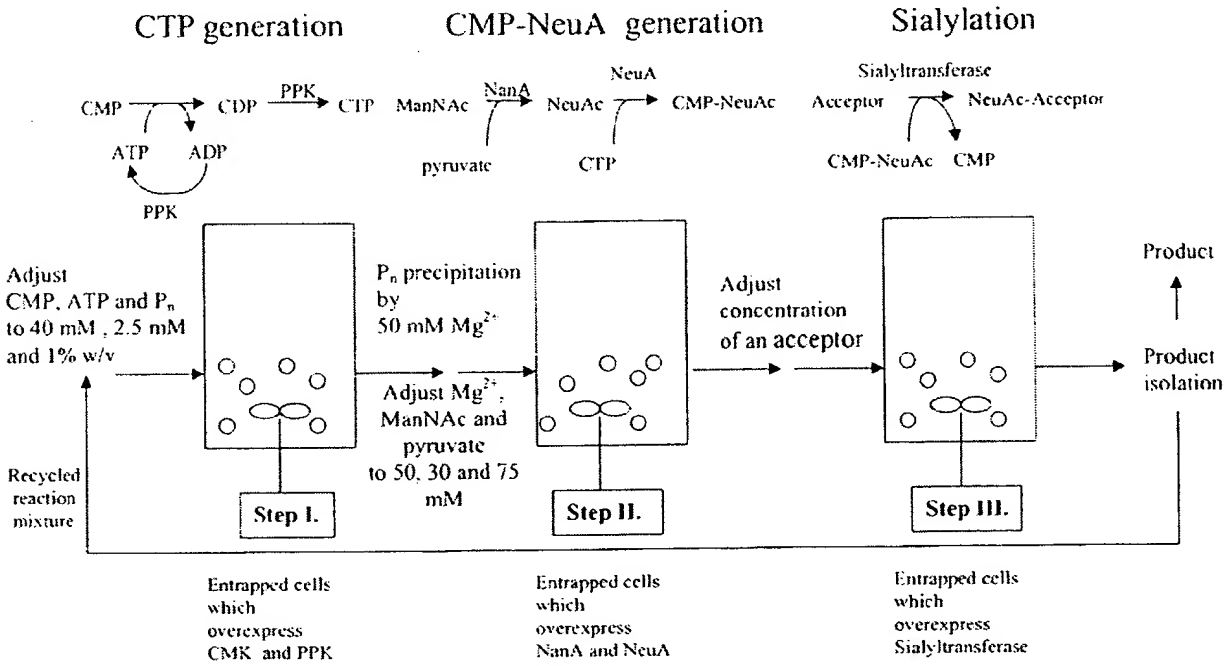


Figure 3 CTP generation from CMP and P_n

◆, CMP; ■, CDP; ▲, CTP; x, cytidine. The reaction was carried out in a volume of 100 ml containing 50 mM Tris/HCl, pH 7.8, 30 mM $MgCl_2$, 0.25 % Triton X-100, 1 % P_n, 40 mM CMP, 2.5 mM ATP, 1 g of P_n kinase *E. coli* fresh cells and 0.5 g of CMK *E. coli* fresh cells.

E. coli BL21(DE3). Since the recombinant proteins have His_6 (hexahistidine) tags, they were easily purified by one-step affinity chromatography and entrapped in PSPS. Elling and co-workers [11,12] demonstrated that the synthesis of nucleotide-sugar with purified enzymes is a better alternative to biotransformation using whole cells, owing to the higher efficiency of product purification. However, the enzymes used have to be isolated and purified, which significantly increases production costs, even if the enzymes are recycled by means of immobilization. Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan) has successfully commercialized nucleotide-sugar production by whole cells [13,14]. From this point of view, we also entrapped *E. coli* cells containing overexpressed enzymes and permeabilized by a freeze–thaw method. Studies comparing the immobilized enzymes (NanA and NeuA) with whole cells harbouring the enzymes were performed. The enzyme preparation (EP) had approximately double the activity [activity at zero time (t_0), 251 munits/g of beads] of the whole-cell preparation (CP; activity at t_0, 103 munits/g of beads). As shown in Figure 2, both preparations showed similar trends in the yield of the CMP-sialic acid repetitive batch synthesis, with an approx. 50% decrease after 15 cycles of usage. When whole cells were used, CTP degradation to cytidine was observed, which resulted in lower efficiency. However, 60–120 g of CP, in contrast with 4–8 g of EP, can be obtained from 1 litre of *E. coli* expression culture. Therefore CP seems to be more economically applicable.

NTP (re)generations for carbohydrate synthesis have been established by combining nucleoside 5′-monophosphate kinases and kinases of high-energy phosphate compounds during the last decade [8,15]. The Kyowa Hakko Company regenerated NTP in nucleotide-sugar production by permeabilized *Corynebacterium ammoniagenes* cells using glucose or fructose as an energy source [13,14]. We have tried both approaches [16], and finally decided to couple recombinant cytidine 5′-monophosphate kinase (CMK) with P_n kinase, because *C. ammoniagenes* gave better results only at low substrate concentration (5 mM) under our conditions. The time course of the reaction showed that there was equilibrium among cytidine, CMP, CDP and CTP up to 10 h (Figure 3). Degradation of CTP to CDP, CMP and cytidine occurred more quickly only after that time. Therefore the conversion cycle was set to finish within 10 h. P_n is a cheap energy source, but it has one disadvantage, namely that it precipitates Mn^{2+} and chelates Mg^{2+} at a concentration of 10–20 mM. Unfortunately, when we coupled CTP generation with CMP-sialic acid synthesis, more than 50 mM Mg^{2+} was needed if Mn^{2+} could not be used at all. One approach to overcome this problem is to continuously monitor and keep the concentration of soluble metal ions constant [17]. However, the easiest and most satisfactory method is to generate CTP first [40 mM CMP, 30 mM $MgCl_2$ and 1 % (w/v) P_n], and then precipitate the remaining P_n with Mn^{2+} (15 mM) or Mg^{2+} (50 mM), followed by CMP-sialic acid synthesis (30 mM ManNAc and 75 mM pyruvate). We routinely obtained 7–11 mM CMP-sialic acid by this approach, and a scheme for sialylation has been proposed (Scheme 1). Separation of CTP and CMP-sialic acid generation into two reaction steps allows one to use cheap P_n as an energy source. Further recycling of the reaction mixture fortified with cytidine decreases CMP degradation by whole cells. We did not couple this with sialylation until now.



Scheme 1 Proposed scheme for sialylation by immobilized biocatalysts

Step I: CTP is generated from CMP, P_n and $MgCl_2$. Step II: CMP-sialic acid ('CMP-NeuAc') is generated from CTP, ManNAc and pyruvate. P_n was precipitated with 50 mM $MgCl_2$ or 15 mM $MnCl_2$, and then ManNAc and pyruvate were adjusted to 30 mM and 75 mM respectively. Step III: the sialylated product is generated after the addition of an acceptor to the mixture.

Conclusions

Use of immobilized biocatalysts in the enzymic synthesis of sugars offers greater productivity, because the enzymes are recycled, and, therefore, downstream enzyme inactivation is not required. The calcium pectate–silica–PEI gel matrix simply mimics the natural pathways for ionotropic gel stability improvement. The procedure is simple, versatile and cheap. The basic advantage lies in the possibility of co-immobilizing enzymes and cells together or preventing the leaking of enzymes from permeabilized cells. It might be argued that this method cannot compete with recombinant-enzyme immobilization via support binding domains (His_6 tag, cellulose-binding domain), which integrates isolation, purification and immobilization procedures. However, from the industrial point of view, whole-cell biotransformation seems to be more applicable, and PSPS or PSPG (pectate–silicate–PEI–glutaraldehyde particles) are suitable for that purpose.

Acknowledgment

The immobilization part of this work was supported by a grant (no. 1/0067/03) from the Slovak Grant Agency for Science, VEGA (Vedecká Grantová Agentúra).

References

1 Gemeiner, P., Kurillová, L'., Markovič, O., Malovíková, A., Uhrín, D., Ilavský, M., Štefuca, V., Polakovič, M. and Báleš, V. (1991) Biotechnol. Appl. Biochem. **13**, 335–345
2 Navrátil, M., Gemeiner, P., Klein, J., Šturdík, E., Malovíková, A., Nahálka, J., Dömény, Z. and Šmogrovičová, D. (2002) Artif. Cells Blood Subst. Immobil. Biotechnol. **30**, 199–218
3 Coradin, T., Mercey, E., Lisnard, L. and Livage, J. (2001) Chem. Commun. **23**, 2496–2497
4 Schneider, S., Feilen, P. J., Slotty, V., Kampfner, D., Preuss, S., Berger, S., Beyer, J. and Pommersheim, R. (2001) Biomaterials **22**, 1961–1970
5 Czichocky, G., Dautzenberg, H., Caplan, E. and Vorlop, K. D. (2001) Biotechnol. Lett. **23**, 1303–1307
6 Fukusima, Y., Okamura, K., Imai, K. and Motai, H. (1988) Biotechnol. Bioeng. **32**, 584–594
7 Morse, M. A. (2000) Curr. Opin. Mol. Ther. **2**, 453–458
8 Ichikawa, Y., Wang, R. and Wong, Ch. H. (1994) Methods Enzymol. **247**, 107–127
9 Kröger, N., Deutzmann, R. and Sumper, M. (2001) J. Biol. Chem. **276**, 26066–26070
10 Kaufman, P. B., Dayanandan, P., Takeoka, Y., Bigelow, W. C., Jones, J. D. and Iler, R. K. (1981) in Silicon and Siliceous Structures in Biological Systems (Simpson, J. L. and Volcani, B. E., eds.), pp. 409–449, Springer-Verlag, New York

11 Zervosen, A. and Elling, L. (1996) J. Am. Chem. Soc. **118**, 1836–1840
12 Zervosen, A., Römer, U. and Elling, L. (1998) J. Mol. Catal. B. Enzym. **5**, 25–28
13 Endo, T. and Koizumi, S. (2000) Curr. Opin. Struct. Biol. **10**, 536–541
14 Endo, T., Koizumi, S., Tabata, K. and Ozaki, A. (2000) Appl. Microbiol. Biotechnol. **53**, 257–261
15 Ishige, K., Hamamoto, T., Shiba, T. and Noguchi, T. (2001) Biosci. Biotechnol. Biochem. **65**, 1736–1740
16 Nahálka, J., Liu, Z., Gemeiner, P. and Wang, P. G. (2002) Biotechnol. Lett. **24**, 925–930
17 Fujio, T. and Furuya, A. (1985) Appl. Microbiol. Biotechnol. **21**, 143–147

Received 8 September 2003/14 November 2003; accepted 19 December 2003
Published as Immediate Publication 19 December 2003,
DOI 10.1042/BA20030159